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13. ABSTRACT *(Maximum 200 words)*

This is a career development award. The primary focus of the research is the expression and function of TGF-β by breast cancer cells. For established tumors, overexpression of TGF-β may result in increased in vivo tumor growth and metastatic spread. The first objective involves the analysis of resected breast cancer specimens, with the goal of confirming our prior work correlating clinical outcome with TGF-β expression levels in tumors. There are as yet no definitive data or conclusions from this aspect of the work. The second objective of the grant was to evaluate the impact of TGF-β on the tumori-genic and metastatic potential of human breast cancer cell lines in nude mice. We have made progress on generating the transfected cell clones, but do not yet have data regarding animal studies. The third research objective is the analysis of the regulation of TGF-β expression in breast cancer cells. We have made some progress in identifying differences in methylation pattern between cells in terms of their usage of the TGF-β3 promoter, and have identified the span of DNA sequence upstream of TGF-β3 which is responsible for the dual promoter usage.

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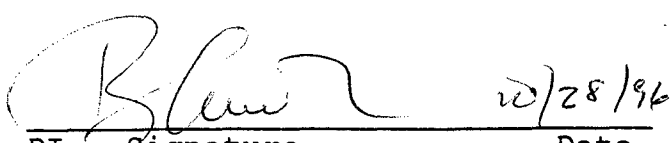

12/28/96

PI - Signature Date

ANNUAL REPORT
Grant Number DAMD17-J-4130
PI: Bradley A. Arrick, M.D., Ph.D.
Institution: Dartmouth College
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Table of Contents

ANNUAL REPORT
Grant Number DAMD17-J-4130
PI: Bradley A. Arrick, M.D., Ph.D.
Institution: Dartmouth College
Reporting Period: 10/1/95-9/30/96

Title: Studies on Human Breast Cancer and Transforming Growth Factor-beta -- Application for a Career Development Award.

Introduction

This is a career development award. The primary focus of the research is the expression and function of TGF-β by breast cancer cells. The specific grant objectives derived from prior work by ourselves and others which have identified TGF-β as an important cytokine in the biology of breast cancer (Gorsch et al. 1992). For established tumors, overexpression of TGF-β may result in increased in vivo tumor growth and metastatic spread. The first objective involves the analysis of resected breast cancer specimens, with the goal of confirming our prior work correlating clinical outcome with TGF-β expression levels in tumors. The second objective of the grant was to evaluate the impact of TGF-β on the tumorigenic and metastatic potential of human breast cancer cell lines in nude mice. The third research objective is the analysis of the regulation of TGF-β expression in breast cancer cells. This primarily deals with identification of the promoter elements responsible for the function of a breast cancer-specific TGF-β3 promoter (Arrick et al. 1994).

One of the purposes of a career development award is to provide the support which would facilitate the awardee's further development of new and hopefully fruitful avenues of research investigation in breast cancer. Working primarily with a graduate student in my laboratory, and collaborating with another laboratory at Dartmouth, we have begun a new project looking into the expression of BRCA1 by breast epithelial cells (BRCA1 being the breast/ovarian cancer susceptibility gene). This work has already produced a publication in which we describe alternative mRNA splicing transcripts (Lu et al., 1996). We hope to be able to continue this line of investigation, with an emphasis upon determining whether the different BRCA1 proteins encoded by these different transcripts have differing functions within breast epithelial cells.

Body of Report

Objective #1

We have no new data to report at this time with regard to the analysis of tissues of patients treated under Cancer and Leukemia Group B clinical protocols for breast cancer. The grant funding this work is not my own, and I have no control over the pace at which other participants in this collaborative effort complete their analysis. I have received assurances from the Statistical office of CALGB that these analyses will be forthcoming shortly after the new year.

Objective #2

We have completed the stable transfection of MDA-MB-231 cells with the dominant negative TGF-β type II receptor (we call these cells "TREZ"). We have documented that they are no longer responsive to the effects of TGF-β, as determined by the transcriptional induction of the plasminogen activator inhibitor I promoter. We are in the final stages of preparing dual transfectants, which we expect will overexpress TGF-β AND be unresponsive to its effects. No data is as yet available for the nude mice studies. This work has been funded by an NIH grant which is currently in its "no cost" extension phase.

Objective #3

#3A

We had been focusing our efforts in working through the many difficulties which we are finding with regard to the reliable preparation from frozen tumor tissue intact mRNA as well as genomic DNA. The preparation of DNA has not been an unsurmountable problem. For this purpose, we have found that grinding the tissue, while still frozen (using liquid nitrogen as cooling source), and then doing an overnight 50°C digestion of the tissue fragments with a mixture of SDS, proteinase K, and EDTA (to inhibit DNase activity), followed by phenol-chlorofoam extraction and ethanol precipitation. This somewhat harsh treatment (which reduces the average size of DNA fragment so-obtained) is of no consequence for later PCR amplification. It is the preparation of high quality mRNA from samples which has continued to plague us. We cannot test, and therefore cannot rule out, the possibility that events which preceed our receipt of the specimen are responsible for the inconsistency we are experiencing. For instance, the amount of time the sample has spent "devitalized", and the temperature of preservation, are not strictly controlled for. At our medical center, mastectomy specimens are not routinely transported to the pathology department on ice, nor are they dissected and processed in the cold.

Progress towards the completion of this objective has fallen behind the timeline of the *Statement of Work* in the grant application We are currently proceeding with the analysis of the extracted genomic DNA from tumor specimens, testing primer pairs for the target gene TGF-β, as well as for a reliable "denominator" genetic locus. If we determine that gene amplification underlies the overexpression phenomenon, we will not need to test the RNA from these samples, and our difficulty in preparing the samples themselves will not hinder our ability to reach an ultimate conclusion for this aim.

#3B

Since submission of our proposal, many other investigators have published analogous work relating to the hormonal control of TGF-β expression in breast cancer cells. We have elected to focus our efforts on the effect of estrogen on TGF-β3, and as discussed

below will delay addressing this question until some of the results from aim 3C are completed. This will allow us to design the appropriate expression plasmids.

#3C

The entirety of this objective is to understand the molecular basis by which breast cancer cells, unlike all other cell types examined by ourselves and others, utilize a different promoter for transcription of the TGF-β3 gene (Arrick et al., 1994). In this report, we will discuss our progress in the analysis of differences in methylation status at the CpG dinucleotides in proximity to the TGF-β3 promoters and our progress with transfections of chimeric TGF-β3 promoter plasmids.

As detailed in our first report, we have not been successful with the experiments described under objective B-1 (DNase hypersensitivity assays). More specifically, when an interpretable result was obtained, there were no convincing differences between cells utilizing both TGF-β3 promoters and those which activate P1 only. This was more fully discussed in last year's report.

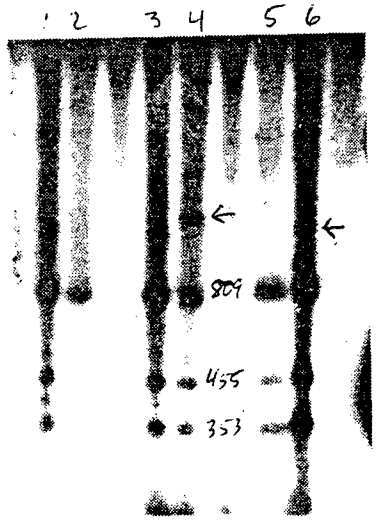
We have conducted experiments as outlined in the proposal looking specifically at the methylation status of HpaII/MspI restriction sites near the TGF-β3 promoters. Figure 1 contains one such experiment. In this experiment, genomic DNA from a breast cancer cell lines which utilizes both TGF-β3 promoters (SKBR3) and two which transcribe TGF-β3 mRNA from the upstream promoter (P1) only (HT-1080 and GBM405). As is outlined in Figure 8 of the original grant proposal, there are two HpaII/MspI sites between the two promoters, one downstream of P2, and a cluster of sites approximately 250-750 basepairs upstream of P1. The probe used in this Southern blot spans the downstream sites and extends to the 5' end of the cluster of sites upstream of P1. Three bands are evident from DNA digested with either enzyme from all three cell lines. These bands are approximately 353, 455, and 809 basepairs in length, and are the predicted bands for restriction enzyme digestion at the sites immediately surrounding the two promoters. In the HpaII lanes from the cell lines which do not transcribe from P2 there is a band evident at about 1.1 kb in length. This is best seen on the gel for HT1080 cells. There is no band of similar size in the HpaII-digested DNA from SKBR3 cells. This presumably reflects differences in methylation at those specific sites. This pattern can arise from either of two possibilities. One possible methylation pattern consistent with these results would be that the 5'-most sites within the cluster of sites are unmethylated in SKBR3 cells but methylated in HT1080 and GBM405 cells (in which case one or more of the remaining sites in this cluster are unmethylated in HT1080 cells and GBM405 cells). It is also possible that the upper band represents the combination of the 809 and 353 bands, which would result if the MspI site closest to the downstream promoter transcription initiation site were partially methylated and therefore somewhat protected from digestion with HpaII (in cells not utilizing that promoter).

Recent publications in the field of CpG methylation patterns have underscored the possibility (indeed likelihood) that at a given site there would be heterogeniety in methylation, even with a pure cell population. In other words, methylation could be

strand specific, and typically when methylated a percentage other than 100% of DNA molecules are so-methylated. Our plan now is to proceed with genomic sequencing utilizing the bisulfite-modification method outlined in the original grant proposal. We will focus our attention on the areas of potential differential methylation suggested by the data such as in Figure 1.

Figure 1.

Southern blot of genomic DNA following digestion with MspI or HpaII -- analysis of CpG methylation upstream of TGF-β3 promoters.



[Legend: DNA was isolated from SKBR3 cells (lanes 1 and 2), HT1080 cells (lanes 3 and 4), and GBM405 cells (lanes 5 and 6). Lanes 2, 4, and 6 were digestion with HpaII, the others with MspI. Arrows point to the bands unique to HpaII digested DNA from the cells which do not utilize P2. The 809, 455, and 353 bp bands are indicated.

Subsequent experiments involved the transfection of cells with chimeric TGF-β3 promoter constructs. In our initial proposal, we provided data (Fig 6 on pg 46) which demonstrated that the DNA spanning the two transcription initiation sites (P1 and P2) had detectable promoter activity in the SKBR3 breast cancer cell line, but not in A673 or HT1080 cells, both of which do not use P2 for transcription of TGF-β3. We have prepared a series of related promoter plasmids which contain 5' or 3' deletions from this putative promoter. Figure 2 contains data from two independent transfection experiments utilizing this panel of plasmids with the SKBR3 cells. A relative activity of 1.0 was assigned to the plasmid containing no deletions (equivalent to the plasmid used in Fig 6 of the original proposal). Removal of the first 175 basepairs from the 5' end resulted in a little over a 5-fold decrease in promoter activity. Between 175 and 491 there is an evident negative element, since some increase in activity is evident with this more extensive deletion. Furthermore, it is apparent that the approximately 200 basepairs closest to P2 are not necessarily essential for transcription. See the brief discussion of objective B-4 for possible significance for the 5'-most 175 basepairs in the regulation of TGF-β3 expression.

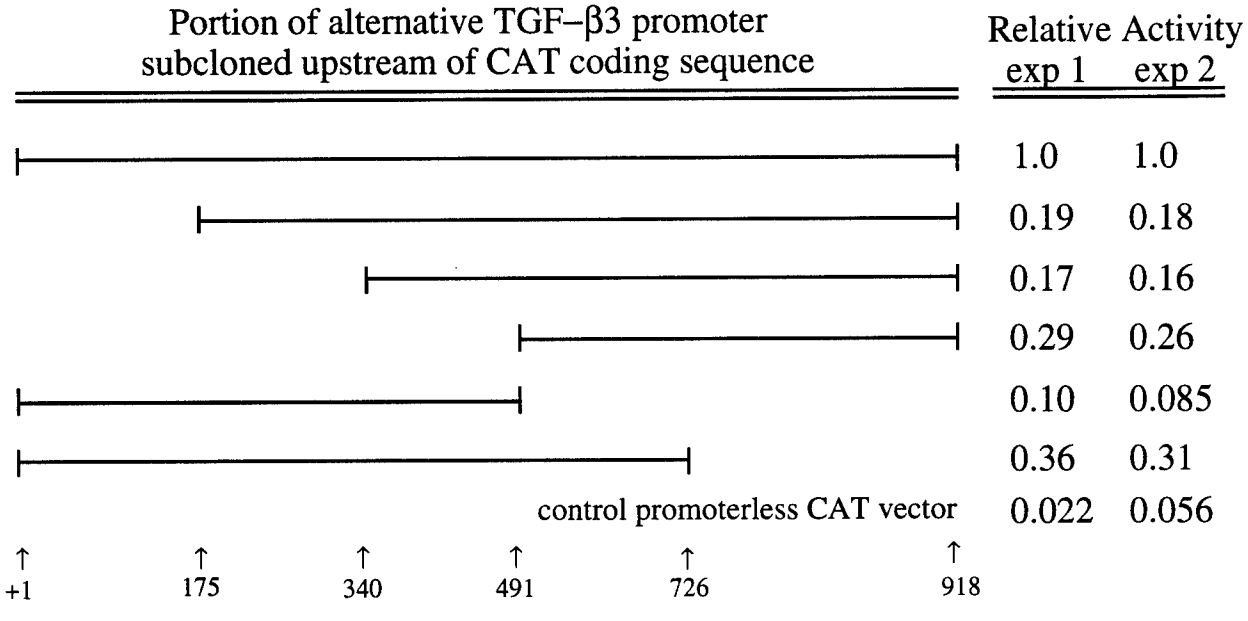


Figure 2.

In last year's report we briefly described that we had begun to prepare stable transfectants using plasmids in which the neomycin resistance gene is under the transcriptional control of the TGF-β3 promoter. G418-resistant colonies were grown up and mRNA isolated. This was then analyzed by Northern analysis, using a neo gene probe. In Figure 3A, from such clones of T47D and SKBR3 cells, we observed predominantly only one transcript species (actual size depends on the exact circumstances of integration into the host cell genome). We then tried a similar

experiment with a plasmid in which the 5'-most 780 base pairs were deleted. This plasmid extends to the FspI site upstream of P1 (refer to diagram on Figure 9, pg 21 of the original proposal). Northern analysis of mRNA isolated from G418-resistant cells transfected with this plasmid (only SKBR3 was successfully transfected this time) demonstrated a recapitulation of the two transcript expression pattern of TGF-β3 in these cells (Figure 3B). This suggests that all of the "information" necessary for the utilization of both P1 and P2 in these cells is contained within this portion of DNA, and is not dependent upon other sequences upstream or downstream of the region included.

Because of the labor intense effort associated with the isolation and characterization of stably transfected cells, we will hold off on further experients of this sort until we define more clearly the methylation pattern differences between unipromoter cells vs bipromoter cells.

Recent work from Yang et al. looking at the hormonal responsiveness of TGF-β3 in osteoblast cells has implicated a small segment of DNA (similar to the 5' most 175 basepairs identified in Fig 2) as important in the induction of TGF-β3 mRNA in these cells in response to estrogen or raloxifen (Yang et al, 1996). Once we have identified the minimal promoter sequence which regenerates the dual promoter expression pattern in breast cancer cells, we will use it, and the plasmid used in the experiment discussed above, to generate a stable transfectant clone from cells which express the estrogen receptor (e.g. T47D or MCF7). Unfortunelty, the SKBR3 cells we have generated do not express the estrogen receptor, and so cannot be used to ask questions related to the estrogen responsiveness of this promoter.

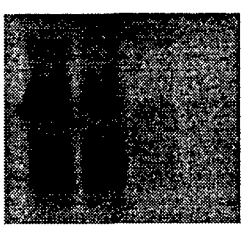
Figure 3A

Northern of stable clones

Legend: Lanes 1 and 2 are from T47D cells, lanes 3 and 4 are from SKBR3 cells. The upper "bands" actually represent cross-hybridization with the large ribosomal subunit RNA.

Figure 3B

Northern of stable clones



Legend: All four lanes are from separate SKBR3 clones. No signal was evident in lane 4, but the dual band pattern seen in lanes 1 and 2 was also evident in lane 3 with longer exposure.

Conclusions

No conclusions can yet be drawn regarding the correlation between TGF-β1 immunoreactivity of patient specimens and clinical outcome. Similarly, no conclusions are possible at this point with respect to objectives #2 and #3A, the tumorigenic and metastatic properties of MDA-MB-231 cells with altered TGF-β production and responsiveness, and examination of patient specimens for mechanisms of TGF-β1 overexpression, respectively.

Work on objective #3C has revealed, in a qualitative sense, a difference in methylation in the region of the TGF-β3 promoter when breast cancer and non-breast cancer cell lines are compared. A more comprehensive mapping of sites of methylation in this region requires that we proceed with the technique of genomic sequencing of bisulfite-modified DNA.

Transient expression studies with chimeric promoter constructs in which the TGF-β3 promoter drives the CAT gene have suggested that there are both positive and negative elements within the first 918 bp of promoter sequence. Specifically, when numbered with nucleotide number 1 being at the site of transcription initiation from the originally-described TGF-β3 promoter, the region spanning 1-175 included a positive element, and a negative element was evident within the region spanning nucleotides 175-491.

Stable transfection studies with SKBR3 cells suggest that all of the elements that direct the utilization of the two TGF-β3 promoters could be contained within the region of DNA flanking these transcription initiation sites. In other words, distant enhancer elemants may not be required.

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Revised Statement of Work -- Covering Years 3 and 4

Objective #1: Confirmation of the prognostic significance of TGF-β expression.

As discussed in the report, there has been some delay in the analysis of a collection of patient specimens from one of the adjuvant breast clinical protocols of the CALGB. Completion of that analysis will occur in year 3. The analysis of further specimens will depend on those results.

Objective #2: Evaluation of the impact of TGF-β on the tumorigenic and metastatic potential of human breast cancer cells in nude mice.

#2A: Stable transfectants which overexpress TGF-β, and other clones which are nonresponsive to TGF-β (because of expression of a dominant negative receptor construct) have been completed. In year 3 we will prepare and characterize double transfected clones (cells which overexpress TGF-β and are nonresponsive to its effects).

#2B: In years 3 and 4 we will conduct the nude mice experiments.

Objective #3: Analysis of the regulation of TGF-β in breast cancer cells.

#3A: As discussed in the report, we have fallen behind in the expected progress towards this goal. In year 3 we will complete analysis for genomic amplification of the TGF-β locus in a representative sample of breast cancer specimens. If amplification is not found in a significant proportion of the samples (e.g >20%), we will reevaluate the scientific validity of this aim, taking into account the results of our immunohistochemical analysis of patient specimens outlined in objective #1.

#3B: We have postponed pursuit of this aim until further progress is made in #3C. Experiments outlined here will not occur until the 4th year of the grant.

#3C: We have been concentrating on (in year 2) and will continue to focus on in year 3 the genomic methylation studies, as described in the report. Data from that work will suggest regions in which to look for DNase hypersensitivity, and the analysis of transfected constructs (year 4). Also in year 4, any important promoter regions identified in the work up to that time will be used as "bait" in looking for DNA-binding proteins.

In summary (timeline):

Year 3: #1 (complete analysis and decide on further specimens to study)
#2A (complete double transfectants)
#2B (nude mouse experiments)
#3A (genomic methylation studies)

Year 4: #1 (depending on results in year 3)
#2B (mouse experiments)
#3A and #3C (specifics depending on results from #3A in year 2)

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 17 Jan 03

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FOR THE COMMANDER:

Phyllis Rinehart

PHYLLIS M. RINEHART
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